(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 731 178 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
12.12.2001 Bulletin 2001/50

(51) Int Cl.[7]: **C12Q 1/68**

(21) Application number: **96301597.9**

(22) Date of filing: **08.03.1996**

(54) **Method of measuring oligonucleotide decomposition**

Verfahren zur Messung von Oligonukleotidabbau

Méthode pour mesurer la décomposition d'un oligonucléotide

(84) Designated Contracting States:
DE FR GB

(30) Priority: **10.03.1995 JP 5092695**

(43) Date of publication of application:
11.09.1996 Bulletin 1996/37

(73) Proprietor: **HAMAMATSU PHOTONICS K.K.**
Shizuoka-ken (JP)

(72) Inventors:
- **Uchiyama, Hisatoshi**
  Hamamatsu-shi, Shizuoka-ken (JP)
- **Jibu, Masaki**
  Hamamatsu-shi, Shizuoka-ken (JP)
- **Hirano, Kenichi**
  Hamamatsu-shi, Shizuoka-ken (JP)
- **Taira, Kazunari**
  Hamamatsu-shi, Shizuoka-ken (JP)

(74) Representative: **West, Alan Harry et al**
R.G.C. Jenkins & Co.
26 Caxton Street
London SW1H 0RJ (GB)

(56) References cited:
EP-A- 0 428 000 **GB-A- 2 278 356**

- **GHOSH ET AL: "Real time kinetics of restriction endonuclease cleavage monitored by fluorescence resonance energy transfer" NUCLEIC ACIDS RESEARCH, vol. 22, no. 15, 1994, pages 3155-3159, XP002054293**
- **CARDULLO R A ET AL: "DETECTION OF NUCLEIC ACID HYBRIDIZATION BY NONRADIATIVE FLUORESCENCE RESONANCE ENERGY TRANSFER" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 85, no. 23, 1 December 1988, WASHINGTON DC, pages 8790-8794, XP000453537**
- **UCHIYAMA AL: "Detection of undegraded oligonucleotides in vivo by fluorescence resonance energy transfer. Nuclease activities in living sea urchin eggs." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 1, 5 January 1996, pages 380-384, XP002054294**

Printed by Jouve, 75001 PARIS (FR)

Description

[0001] The present invention relates to an in vitro method of measuring the decomposition of oligonucleotides.

[0002] An effective means for studying the control of the expression of a target gene, has been to inject a functional oligonucleotide into a cell of an organism to see how this oligonucleotide behaves within the cell (Erickson, R.P., and Izant, J.G. ed., 1992, Gene Regulation: Biology of Antisense RNA and DNA, Raven Press, New York; Murray, J.A.H. ed., 1992, Antisense RNA and DNA, Wiley-Liss, New York).

[0003] For example, it is known that, when injected into a cell, short DNA molecules combine with their corresponding mRNAs at sites of base sequence complementarity thereby inhibiting the synthesis of a particular protein (Wagner, R. W., 1994, Nature 372, 333-335). In the therapeutic study of AIDS, a specifically designed ribozyme has been known to cut the mRNA of the AIDS virus (Sarver, N., et al., Science 247, 1222-1225, 1990), suggesting that ribozymes and their synthetically modified analogues are candidates for useful drugs in the future.

[0004] The stability or reliability of these functional oligonucleotides within an organism largely depends on their base sequence and length, which affect their susceptibility to the various biological materials such as nucleases existing within an organisms cells.

[0005] Accordingly, there is a strong possibility that a functional oligonucleotide injected into an organism will be cut (or decomposed), for example, by a nuclease within a cell. Using conventional techniques, it has been very difficult to detect whether the target oligonucleotide has been decomposed or not.

[0006] Conventionally, in order to monitor an oligonucleotide within an organism, investigators have used a probe made of an oligonucleotide in which one end is labelled with a fluorescent dye.

[0007] However, in this technique, there is a strong possibility that, even when the oligonucleotide probe has lost its function due to a decomposition reaction such as nucleolysis by a nuclease, the fluorescence of the dye in a decomposed fragment can still be detected.

[0008] Accordingly, the inventors have diligently conducted studies to overcome the foregoing problems. They have succeeded in developing a method by which changes in the molecular structures of a subject oligonucleotide within a cell, such as its decomposition caused by a nuclease or the like, can be detected with high sensitivity. Then, based on this method, the inventors have succeeded in providing a method by which the stability or reliability of the subject oligonucleotide can be evaluated in vitro.

[0009] The in vitro method of measuring the decomposition of a single-stranded oligonucleotide in accordance with the present invention comprises the steps of labelling the oligonucleotide with two different fluorescent dyes (or fluorochromes), and then inspecting a fluorescence characteristic based on an intramolecular fluorescence resonance energy transfer (FRET) [Stryer, L. (1978), Annu. Rev. Biochem., 47, 819-846.; Herman, B. (1989) In Taylor, D.L. and Wang, Y. (eds.), Fluorescence Microscopy of Living Cells in Culture-Part B. Academic Press, New York, pp. 220-245.] caused by these two kinds of fluorochromes.

[0010] More specifically, the present invention provides an in vitro method of measuring the decomposition of a single-stranded (or single chain) oligonucleotide in which the measurement is performed according to the detection of a fluorescence resonance energy transfer caused by an energy donor group containing a fluoroscein fluorochrome and an energy acceptor group containing a rhodamine fluorochrome combined with the 5'- and 3'-terminals of the oligonucleotide.

[0011] The invention provides an in vitro measuring method for single-stranded oligonucleotides which comprise not less than 3 but not more than 20 nucleotides. More particularly, the present invention provides a measuring method for single-stranded oligonucleotide comprising 10 nucleotides.

[0012] Preferably, the energy donor group and the energy acceptor group are combined with the 5'- and 3'-terminals of the oligonucleotide, in which the energy donor group contains a fluorescein fluorochrome and the energy acceptor group contains at least a rhodamine fluorochrome.

[0013] The present invention will be more fully understood from the detailed description given below and the accompanying drawings, which are given by way of illustration only and are not to be considered as limiting the present invention.

[0014] Further scope of applicability of the present invention will become apparent from the detailed description given below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will be apparent to those skilled in the art from this detailed description.

Fig. 1 is a schematic view illustrating the principle underlying the present invention, in which the FRET observed on the basis of the relationship between the energy donor (D) and energy acceptor (A) combined with a subject single-stranded oligonucleotide becomes unobservable due to a decomposition reaction;

Fig. 2 is a chart showing a high-pressure liquid chromatogram of 5'-fluorescein-TGAAATTGTT-3'-rhodamine X (F-ODN-R) after purification and separation;

Fig. 3 is a chart showing an absorption spectrum of 5'-fluorescein-TGAAATTGTT-3'-rhodamine X (F-ODN-R) ;
Fig. 4 is a chart showing an emission spectrum of 5'-fluorescein-TGAAATTGTT-3'-rhodamine X (F-ODN-R) exited at 494 nm;
Fig. 5 is a chart showing an excitation spectrum of 5'-fluorescein-TGAAATTGTT-3'-rhodamine X (F-ODN-R) monitored at 606 nm;
Fig. 6 is a chart showing (as continuous curve) an emission spectrum of 5'-fluorescein-TGAAATTGTT-3'-rhodamine X (F-ODN-R) before (solid line) and after (dotted line) enzymatic digestion with BAL31;
Fig. 7 is a chart showing the molecular structure of 5'-rhodamine-TGAAATTGTU-3'-fluorescein;
Fig. 8 is a chart showing the molecular structure of 5'-fluorescein-TGAAATTGTT-3'-rhodamine X;
Fig. 9 is a schematic view illustrating the operation of the present invention, in which a subject oligonucleotide by which the FRET becomes observable is injected into a cell and then the change in the FRET within the cell is monitored so as to trace the decomposition reaction caused by nucleases within the cell; and
Fig. 10A is a chart showing changes in the ratio of two fluorescence components, i.e., red component/green component, from sea urchin eggs over time under a fluorescence microscope, which shows the change in the ratio in a sea urchin egg into which R-ODN-F (having a natural phosphodiester linkage) has been injected.
Fig. 10B is a chart showing the change in the ratio in a sea urchin egg into which R-S-ODN-F (having a nuclease-resistant phosphorothioate linkage) has been injected.

Decomposition of a Single-Stranded Oligonucleotide

[0015] In the present invention, the decomposing activity against a subject single-stranded oligonucleotide refers to the ability to cleave a single-stranded oligonucleotide by decomposition reactions under various conditions in vitro, thereby inactivating it. For example, a single-stranded oligonucleotide subjected to a single hydrolysis reaction will be divided into two. Regardless of whether or not the decomposing portion has been known beforehand, the single-stranded oligonucleotide can be used in the present invention without any restriction. In cases where the decomposing portion has been known beforehand, it can be further confirmed whether or not such a portion has been subjected to such a reaction as cutting. In general, physiological activities observed in an oligonucleotide are lost or become very weak when at least a part of the oligonucleotide is removed. Accordingly, it is important to know of any changes due to decomposition and, in particular, to monitor these against real time.

Fluorescence Resonance Energy Transfer, FRET

[0016] The FRET in the present invention is a fluorescence phenomenon described in the literature (e.g., Stryer, L. Ann. Rev. Biochem., 47, 1978, 819-846; In Taylor, D.L. and Wang, Y. eds., Fluorescence Microscopy of Living Cells in Culture-Part B. Academic Press, New York, 220-245, 1989). In short, when (1) the fluorescence spectrum of one fluorogen (energy donor) overlaps with the excitation spectrum of the other fluorogen (energy acceptor) and (2) the donor and the acceptor are positioned close to each other, the excitation of the donor induces the fluorescence from the acceptor while reducing the fluorescence intensity resulting from the donor itself. The phenomenon is referred to as FRET.

[0017] FRET is known to be quite sensitive to the distance between the donor and the acceptor and, in general, proportional to 10^{-6} of this distance.

[0018] Accordingly, when a donor and acceptor pair providing the FRET is disposed in a subject and the disappearance of the FRET occurring between the donor and the acceptor is monitored, it can be deduced that the subject has suffered a great change in the distance between the donor and the acceptor, namely, a portion containing the donor group and acceptor group has been cut, for example, and is not in a single molecule any more (Fig 1).

Energy Donor and Energy Acceptor

[0019] A dye having a fluorescein fluorogen and a dye having a rhodamine X fluorogen are used as the donor and the acceptor, respectively. The absorption spectra of fluorescein and rhodamine X have absorption peaks at 497 nm and 586 nm, respectively. When excited at 494 nm, fluorescein exhibits a fluorescence peak at 523 nm. Whilst rhodamine X exhibits a fluorescence peak at 610 nm when excited at 585 nm.

[0020] In cases where there is an appropriate distance between the two kinds of fluorochromes for generating the FRET, as the fluorescence energy transfers from fluorescein which is the donor to rhodamine X which is the acceptor, the fluorescence intensity derived from fluorescein decreases while that derived from rhodamine X increases.

Preparation of Single-Stranded Oligonucleotide

[0021] The nature of the base sequence of the single-chain oligonucleotide can be selected according to its purpose as no special restrictions are imposed on base sequence by the present invention.

[0022] In order to utilize the FRET in the present invention, it is necessary for two kinds of fluorochromes to be disposed with a distance therebetween being within a certain preferable range. The number of base sequences in the oligonucleotide between these two kinds of fluorochromes is dependent on the selection of the fluorochromes which can utilize the FRET.

[0023] Accordingly, it is preferable to prepare a subject oligonucleotide such that the two kinds of fluorochromes are sited at the appropriate portions thereof (holding therebetween a portion which will be cut upon a decomposition reaction). For example, when fluorescein and rhodamine X are used as the fluorochromes, the number of base sequences in the oligonucleotide therebetween is most preferably about 10. More generally, the oligonucleotide therebetween preferably comprises not less than 3 but not more than 20 nucleotides. When it is too short, the position where the subject is decomposed may be quite limited; whereas, when it is too long, the FRET may not be observed sufficiently. Under these circumstances, the preferred range comprises not less than 5 but not more than 18 (or, more preferably, not less than 8 but not more than 15) nucleotides.

[0024] It is easy for one skilled in the art to synthesize an oligonucleotide having such a number of base sequences so as to include a position where a subject is decomposed, to select appropriate fluorochromes, and then to combine these fluorochromes with the oligonucleotide portion to prepare a subject oligonucleotide.

[0025] In the present invention, other than the oligonucleotide portion that is subjected to a decomposition reaction, there is no restriction about the molecular composition as long as this reaction is not influenced thereby.

[0026] Accordingly, groups such as those of an oligonucleotide or oligopeptide may be further combined with the 5'- or 3'-terminals of the subject oligonucleotide that is subjected to decomposition. Namely, in one embodiment of the invention, the oligonucleotide of the above-mentioned predetermined number of bases including an estimated decomposing position of a single functional polynucleotide is selected and the above-mentioned fluorochromes by which the FRET can be utilized are combined with both ends thereof, thereby making it possible to trace a reaction in which such the position is decomposed. In this case, except for the above-mentioned polynucleotide portion, there is substantially no restriction.

[0027] Further, in the present invention, it is not always necessary for the subject to be a single chain at the time of being decomposed. It may form a double chain with other complementary DNAs, for example, before being subjected to the decomposition reaction, whereby the above-mentioned change in FRET can be observed.

[0028] There is no restriction concerning the method by which the subject oligonucleotide is synthesized. Any known method for synthesizing nucleotides may be suitably used. For example, chemical synthesis methods or automatic synthesis methods may be used. Synthesis methods using enzymes may also be suitable.

Preparation of Subject Oligonucleotide Having Fluorochromes

[0029] There is no restriction concerning the method of combining the energy donor group and the energy acceptor group with the 5'- and 3'-terminals of the oligonucleotide portion. General organic synthesis methods or enzymatic reactions using appropriate derivatives may be used to prepare the subject oligonucleotide having fluorescence in various forms.

(1) A desirable energy donor group and an energy acceptor group such as those described above are combined with a target oligonucleotide. The target oligonucleotide may be synthesized first and then active groups at its 5'- and 3'-terminals (e.g., their OH or NH2 groups) may be combined with the energy donor groups and energy acceptor group by known chemical reactions. A linker portion may be additionally inserted between the energy donor and receptor groups to be introduced and the terminal groups in order to attain an appropriate length.

(2) A double-labelled oligonucleotide is synthesized using two kinds of fluorescently labelled mononucleotides as the substrates of the starting and ending reactions of the synthesis in order to incorporate fluorochromes at both terminals of the oligonucleotide as the energy donor and acceptor.

[0030] In examples of the present invention, a fluorescein fluorochrome was combined with the 5'-terminal of an oligonucleotide (10-mer) having a rhodamine X fluorochrome at its 3'-terminal by a chemical synthesis method.

[0031] Similarly, in the present invention, a mononucleotide having a fluorescein fluorochrome was combined with the 3'-terminal of an oligonucleotide (9-mer) having a rhodamine X fluorogen at its 5'-terminal by an enzymatic chemical method.

Measurement of Decomposing Activity by FRET Measurement

[0032] In the present invention, any method may be used to measure fluorescence spectra derived from two kinds of fluorochromes and to compare them with each other.
[0033] For example, FRET may be estimated by comparison between the ratio of the fluorescence intensity values derived from each of two fluorochromes under conditions in which the FRET is possible and the ratio of the fluorescence intensity values derived from each fluorochromes under conditions in which the FRET does not occur.
[0034] In order to measure the FRET efficiency with higher sensitivity, selection of excitation wavelength, filters for selective measurement of fluorescence components, and means for simultaneously measuring two kinds of fluorescence components, may be used.
[0035] Also, when a phenomenon based on the FRET is influential to other phenomenons such as a change in the life-time of fluorescence, such a change in the life-time can be utilized in the measurement of decomposition in accordance with the present invention.
[0036] Further, in the present invention, any nucleolytic activity of enzymes can be estimated, regardless of whether it decomposes a single-stranded oligonucleotide or double-stranded oligonucleotide.

Measurement of Decomposing Activity by FRET Measurement in Vivo

[0037] In the method of measuring decomposition of an oligonucleotide in accordance with the present invention, there is no restriction concerning the measuring method in vivo.
[0038] Any means may be used as long as it can measure the fluorescence values derived from two kinds of fluorochromes. For example, while a sample is observed with a fluorescence microscope, two kinds of filters may be used to measure the fluorescence values derived from the two kinds of fluorochromes and monitor their changes with time.
[0039] In the present invention, for example, an oligonucleotide (R-ODN-F) labelled with two kinds of fluorogens may be injected into a sea urchin egg such that its FRET can be observed in vivo.
[0040] As the FRET is observed, it can be confirmed in vivo that the oligonucleotide is not decomposed in a cell.
[0041] Also, when the FRET changes temporally time or according to other biochemical processings, the decomposing state of the oligonucleotide can be observed on real time.
[0042] The foregoing techniques provide means for measuring the decomposing activity of various kinds of nucleases against an oligonucleotide in vitro or in vivo.
[0043] Also, the stability and life-time of the oligonucleotide, for example, in a cell can be monitored against real time.
[0044] In a preferred embodiment the FRET measurement systems under a microscope comprise a fluorescence microscope, the separation optics of fluorescence components, photo-detectors such as high sensitivity video cameras, and a computer for data analysis such as an image processor, where the fluorescence light from the microscope is separated into red and green components through the separation optics filters(A) or by a dichroic mirror. In the present example of the invention described below, the red and green components of fluorescence from the same egg were separated through filters and accumulated with a high-sensitivity video camera. The fluorescence intensity was analyzed with a computer.
[0045] The present invention will now be explained in detail with reference to the examples. Nevertheless, the present invention should not be restricted to these examples.

Example 1

Synthesis of 5'-Rhodamine X-TGAAATTGTU-3'-Fluorescein (R-ODN-F) (Fig. 7)

[0046] R-ODN-F was synthesized from 3'-fluorescein-1,2-dideoxyuridine-5'-triphosphate (ddUTP-F) (manufactured by Boehringer Mannheim) and 5'-rhodamine X-TGAAATTGT-3' (R-ODN) (manufactured by Takara Shuzo) using the following enzymatic reaction.
[0047] 0.1 mM of ddUTP-F and 0.05 mM of R-ODN were incubated with 20 units of terminal deoxynucleotidyl transferase (manufactured by GIBCO-BRL) in a mixture made of 100-mM cacodylic acid potassium buffer solution (pH 7.2), 2 mM of $CoCl_2$, and 1 mM of dithiothreitol (23) for 2 hours at 37°C.
[0048] The resulting R-ODN-F was purified and separated by a high-pressure liquid chromatography (HPLC, model 8100, manufactured by Tosoh) using an ion-exchange column (TSK gel DEAE-5PW, manufactured by Tosoh) under the following conditions:

Flow rate	1.0 ml/min
Temperature	room temperature 25°C

(continued)

Solvent	linear concentration gradient using 0.1 M to 1 M of NaCl in 20-mM Tris-HCl buffer solution (pH 9.0)

Example 2

Synthesis of 5'-Fluorescein-TGAAATTGTT-3'-Rhodamine X (F-ODN-R) (Fig. 8)

[0049] It was synthesized by a chemical synthesis method and a single peak was confirmed therein using HPLC (Fig. 2).

Absorption Spectrum and Fluorescence Spectrum

[0050] F-ODN-R was diluted to an appropriate concentration by solvent A (50-mM Tris-HCl buffer solution at pH 8.0, 0.1 M of NaCl, 1 mM of $CaCl_2$, and 1 mM of $MgCl_2$).

[0051] Its absorption spectrum and fluorescence spectrum were obtained by a spectrophotometer (Hitachi model 557) and a fluorescence spectrophotometer (Hitachi model 850), respectively.

[0052] As shown in Fig. 3, the absorption spectrum of F-ODN-R coincides with an absorption spectrum obtained when respective absorption spectra of fluorescein and rhodamine X are superimposed on each other.

[0053] Fig. 4 shows a fluorescence spectrum of F-ODN-R.

[0054] When excited at 494 nm, it exhibits fluorescence peaks at 523 nm and 610 nm which correspond to respective fluorescence spectra of fluorescein and rhodamine X superimposed on each other.

[0055] Similarly, when monitored at 606 nm, it provides excitation peaks at 500 nm and 594 nm (Fig. 5).

[0056] When their respective fluorescence intensity values are compared, it is found that, upon excitation at 494 nm, the intensity of the fluorescence spectrum derived from rhodamine X at 610 nm is several times as much as that derived from fluorescein.

[0057] This result suggests that the excitation energy of the fluorescein fluorogen has transferred to the rhodamine fluorogen, thereby changing their fluorescence intensity values.

Digestion of Oligonucleotide by Enzyme

[0058] Thus obtained oligonucleotide (F-ODN-R), as a substrate, was digested by BAL31 nuclease (manufactured by Takara Shuzo) having a function as endonuclease for single-chain DNAs (Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring, 1989).

[0059] For example, measurement in vitro was performed after the F-ODN-R (17 nM) in 2 ml of solvent A had been incubated with 5 units of BAL31 at 30°C for 60 minutes.

[0060] Alternatively, measurement in vivo was performed after the F-ODN-R (50 μM) in 4 μl of solvent A had been incubated with 0.1 unit of BAL31 at 30°C for 30 minutes.

[0061] The digestion by this enzyme was stopped when 2 μl of 0.1-M EGTA was added to 4 μl of the reaction solution.

Sea Urchin Eggs and Microinjection

[0062] Eggs of a sea urchin (Hemicentrotus pulcherrimus) were collected as 0.55-M KCl was injected into its body cavity and then washed twice with seawater. The eggs were disposed on a poly-L-lysine-coated glass cover slip (with frames made of Lucite forming a chamber having a capacity of about 3 ml).

[0063] The oligonucleotide was suspended to a concentration of 100 μM in 100-mM HEPES-KOH at pH 7.2. The oligonucleotide solution (about 1 to 2% with respect to the egg volume) was microinjected into the eggs (Hirano, K. Develop. Growth and Differ. 24, 1982, 273-281).

Visualization of FRET under Microscope

[0064] In order to observe the FRET phenomenons resulting from the sea urchin eggs, 2 filters provided for a fluorescence microscope (Diaphoto-TMD, manufactured by Nikon) were respectively used for observing the fluorescence components derived form the injected oligonucleotide.

[0065] Green images were observed through a band-pass filter of 520 to 560 nm, whereas red images were observed through a sharp-cut filter of 580 nm and over.

[0066] A heat-absorbing filter was used to eliminate infrared rays from both of these types of images.

[0067] The excited light obtained through a band-pass filter of 470 to 490 nm was eliminated from the observed

fluorescence signal (with a cut-off wavelength of 510 nm by a dichroic mirror).

[0068] Thus obtained green and red images were captured by a highly sensitive ICCD camera (c2400-80, manufactured by Hamamatsu Photonics) and the fluorescence intensity was processed and analyzed with an image processor (ARGUS-50, manufactured by Hamamatsu Photonics).

[0069] The sea urchin egg to which the oligonucleotide had been microinjected exhibited bright red fluorescence in addition to the green fluorescence derived from fluorescein.

[0070] In contrast, no red fluorescence but only green fluorescence was observed in the sea urchin egg into which the BAL31-digested product had been injected.

[0071] The fluorescence intensity varied in different parts of the egg. Namely, the intensity was stronger at the center of the egg, while both green and red were weaker at the periphery. This problem resulted from differences in optical path length and could be corrected when the cell volume was amended.

Claims

1. An in vitro method of monitoring the cleavage of a single-stranded oligonucleotide, comprising the steps of;

a) detecting a resonance energy transfer caused by an energy donor group containing a fluorescein fluorochrome and an energy acceptor group containing a rhodamine fluorochrome combined with the oligonucleotide, and

b) detecting a change in the resonance energy transfer resulting from decomposition of the oligonucleotide between the energy donor group and the energy acceptor group.

2. A method according to claim 1, wherein the energy donor group and the energy acceptor group are combined with the 5'- and 3'-terminals of the oligonuclectide.

3. A method according to claim 1 or claim 2, wherein the fluorescence energy transfer (FRET) is detected.

4. A method according to any preceding claim, wherein the oligonucleotide between the energy donor group and the energy acceptor group comprises not less than 3 but not more than 20 nucleotides.

5. A method according to any one of claims 1 to 3, wherein the oligonucleotide between the energy donor group and the energy acceptor group comprises 10 nucleotides.

6. An in vitro method of measuring a decomposing activity against a subject single-stranded oligonucleotide comprising the steps of detecting a resonance energy transfer caused by a set of energy donor group and energy acceptor group combined with said subject oligonucleotide and then detecting a change in said resonance energy transfer resulting from a decomposition reaction of an oligonucleotide chain between said set of energy donor group and energy acceptor group.

7. An in vitro method of measuring a decomposing activity against a subject single-stranded oligonucleotide comprising the steps of detecting a resonance energy transfer caused by a set of energy donor group and energy acceptor group combined with 5'- and 3'-terminals of said subject oligonucleotide and then detecting a change in said resonance energy transfer resulting from a decomposition reaction of said oligonucleotide.

8. An in vitro method of measuring a decomposing activity against a subject single-stranded oligonucleotide comprising the steps of detecting a fluorescence resonance energy transfer (FRET) caused by a set of energy donor group and energy acceptor group combined with said subject oligonucleotide and then detecting a change in said fluorescence resonance energy transfer (FRET) resulting from a decomposition reaction of an oligonucleotide chain between said set of energy donor group and energy acceptor group.

9. An in vitro method of measuring a decomposing activity against a subject single-stranded oligonucleotide comprising the steps of detecting a fluorescence resonance energy transfer (FRET) caused by a set of energy donor group and energy acceptor group combined with 5'- and 3'-terminals of said subject oligonucleotide and then detecting a change in said fluorescence resonance energy transfer (FRET) resulting from a decomposition reaction of said oligonucleotide.

Patentansprüche

1. In vitro-Verfahren zum Aufzeichnen des Abbaus eines einzel-strängigen Oligonukleotids, umfassend die Schritte:

   a) Detektion eines Resonanz-Energietransfers, der verursacht wird durch eine ein Fluorescein-Fluorochrom enthaltende Energiedonorgruppe und eine ein Rhodamin-Fluorochrom enthaltende Energieakzeptorgruppe, die mit dem Oligonukleotid verbunden sind und
   b) Detektion einer Veränderung im Resonanz-Energietransfer, die aus dem Abbau des Oligonukleotids zwischen der Energiedonorgruppe und der Energieakzeptorgruppe resultiert.

2. Verfahren gemäß Anspruch 1, wobei die Energiedonorgruppe und die Energieakzeptorgruppe mit den 5'- und 3'-Enden des Oligonukleotids verbunden sind.

3. Verfahren gemäß Anspruch 1 oder 2, wobei der Fluoreszenz-Energietransfer (FRET) detektiert wird.

4. Verfahren gemäß irgendeinem der vorangehenden Ansprüche, wobei das Oligonukleotid zwischen der Energiedonorgruppe und der Energieakzeptorgruppe nicht weniger als 3, jedoch nicht mehr als 20 Nukleotide aufweist.

5. Verfahren gemäß irgendeinem der Ansprüche 1 bis 3, wobei das Oligonukleotid zwischen der Energiedonorgruppe und der Energieakzeptorgruppe 10 Nukleotide aufweist.

6. In vitro-Verfahren zum messen der Abbauaktivität gegenüber einem gegenständlichen, einzel-strängigen Oligonukleotid, das die Schritte des Detektierens eines Resonanz-Energietransfers, der durch einen Satz einer Energiedonorgruppe und einer Energieakzeptorgruppe verursacht wird, die mit dem gegenständlichen Oligonukleotid verbunden sind, und des anschließenden Detektierens einer Veränderung in dem Resonanz-Energietransfer, die aus der Abbaureaktion einer Oligonukleotidkette zwischen dem Satz der Energiedonorgruppe und Energieakzeptorgruppe resultiert, umfasst.

7. In vitro-Verfahren zum Messen der Abbauaktivität gegenüber einem gegenständlichen, einzel-strängigen Oligonukleotid, welches die Schritte des Detektierens eines Resonanz-Energietransfers, der durch einen Satz einer Energiedonorgruppe und einer Energieakzeptorgruppe verursacht wird, die mit dem 5'- und 3'-Enden des gegenständlichen Oligonukleotids verbunden sind, und des Detektierens der Veränderung im Resonanz-Energietransfer, die aus der Abbaureaktion des Oligonukleotids resultiert, umfasst.

8. In vitro-Verfahren zum Messen der Abbauaktivität gegenüber einem gegenständlichen, einzel-strängigen Oligonukleotid, welches die Schritte des Detektierens eines Fluoreszenz-Resonanz-Energietransfers (FRET), der durch einen Satz einer Energiedonorgruppe und einer Energieakzeptorgruppe verursacht wird, die mit dem gegenstädnlichen Oligonukleotid verbunden sind, und des Detektierens der Veränderung im Fluoreszenz-Resonanz-Energietransfer (FRET), die aus einer Abbaureaktion einer Oligonukleotidkette zwischen dem Satz der Energiedonorgruppe und Energieakzeptorgruppe resultiert, umfasst.

9. In vitro-Verfahren zum Messen der Abbauaktivität gegenüber einem gegenständlichen, einzel-strängigen Oligonukleotid, welches die Schritte des Detektierens eines Fluoreszenz-Resonanz-Energietransfers (FRET), der durch einen Satz einer Energiedonorgruppe und einer Energieakzeptorgruppe verursacht wird, die mit dem 5'- und 3'-Enden des gegenständlichen Oligonukleotids verbunden sind, und des Detektierens der Veränderung im Fluoreszenz-Resonanz-Energietransfers (FRET), die aus einer Abbaureaktion des Oligonukleotids resultiert, umfasst.

Revendications

1. Procédé *in vitro* de contrôle du clivage d'un oligonucléotide monobrin, comprenant les étapes consistant ;

   a) à détecter un transfert d'énergie de résonance provoqué par un groupe donneur d'énergie contenant un fluorochrome fluorescéine et un groupe accepteur d'énergie contenant un fluorochrome rhodamine combinés avec l'oligonucléotide, et
   b) à détecter un changement dans le transfert d'énergie de résonance provenant de la décomposition de l'oligonucléotide entre le groupe donneur d'énergie et le groupe accepteur d'énergie.

2. Procédé selon la revendication 1, dans lequel le groupe donneur d'énergie et le groupe accepteur d'énergie sont combinés avec les extrémités 5' et 3' de l'oligonucléotide.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel le transfert d'énergie de fluorescence (FRET) est détecté.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'oligonucléotide compris entre le groupe donneur d'énergie et le groupe accepteur d'énergie ne comprend pas moins de 3 mais pas plus de 20 nucléotides.

5. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel l'oligonucléotide compris entre le groupe donneur d'énergie et le groupe accepteur d'énergie comprend 10 nucléotides.

6. Procédé *in vitro* de mesure d'une activité de décomposition envers un oligonucléotide monobrin sujet comprenant les étapes consistant à détecter un transfert d'énergie de résonance provoqué par un jeu d'un groupe donneur d'énergie et d'un groupe accepteur d'énergie combinés avec ledit oligonucléotide sujet, puis à détecter un changement dans ledit transfert d'énergie de résonance résultant d'une réaction de décomposition d'une chaîne oligonucléotide comprise entre le groupe donneur d'énergie et le groupe accepteur d'énergie dudit jeu.

7. Procédé *in vitro* de mesure d'une activité de décomposition envers un oligonucléotide monobrin sujet comprenant les étapes consistant à détecter un transfert d'énergie de résonance provoqué par un jeu d'un groupe donneur d'énergie et d'un groupe accepteur d'énergie combinés avec les extrémités 5' et 3' dudit oligonucléotide sujet, puis à détecter un changement dans ledit transfert d'énergie de résonance provenant d'une réaction de décomposition dudit oligonucléotide.

8. Procédé *in vitro* de mesure d'une activité de décomposition envers un oligonucléotide monobrin sujet comprenant les étapes consistant à détecter un transfert d'énergie de résonance de fluorescence (FRET) provoqué par un jeu d'un groupe donneur d'énergie et d'un groupe accepteur d'énergie combinés avec ledit oligonucléotide sujet puis à détecter un changement dans ledit transfert d'énergie de résonance de fluorescence (FRET) résultant d'une réaction de décomposition d'une chaîne oligonucléotide comprise entre le groupe donneur d'énergie et le groupe accepteur d'énergie dudit jeu.

9. Procédé *in vitro* de mesure d'une activité de décomposition envers un oligonucléotide monobrin sujet comprenant les étapes consistant à détecter un transfert d'énergie de résonance de fluorescence (FRET) provoqué par un jeu d'un groupe donneur d'énergie et d'un groupe accepteur d'énergie combinés avec les extrémités 5' et 3' dudit oligonucléotide sujet, puis à détecter un changement dans ledit transfert d'énergie de résonance de fluorescence (FRET) résultant d'une réaction de décomposition dudit oligonucléotide.

Fig. 1

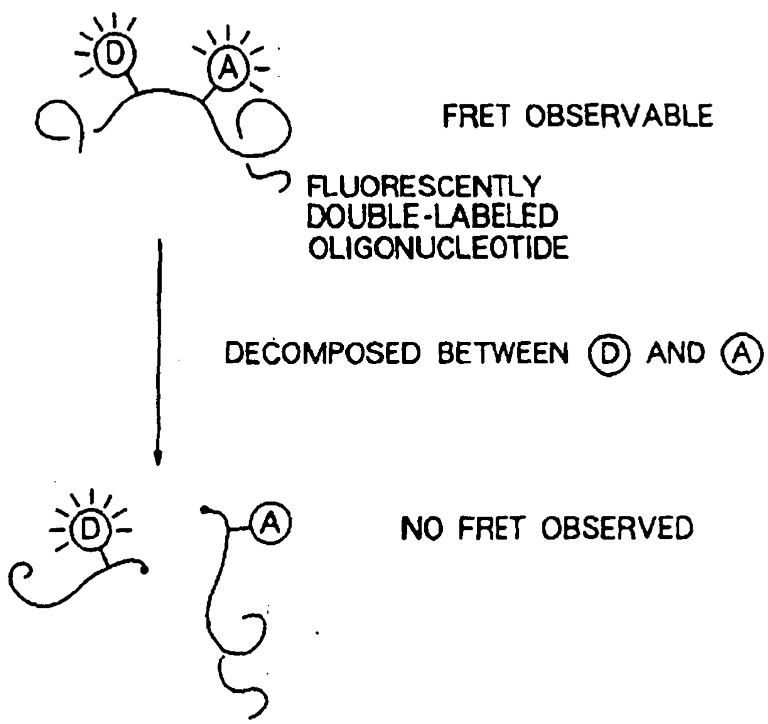
D
A
FRET OBSERVABLE
FLUORESCENTLY
DOUBLE-LABELED
OLIGONUCLEOTIDE
DECOMPOSED BETWEEN D AND A.
D
A
NO FRET OBSERVED

Fig. 2

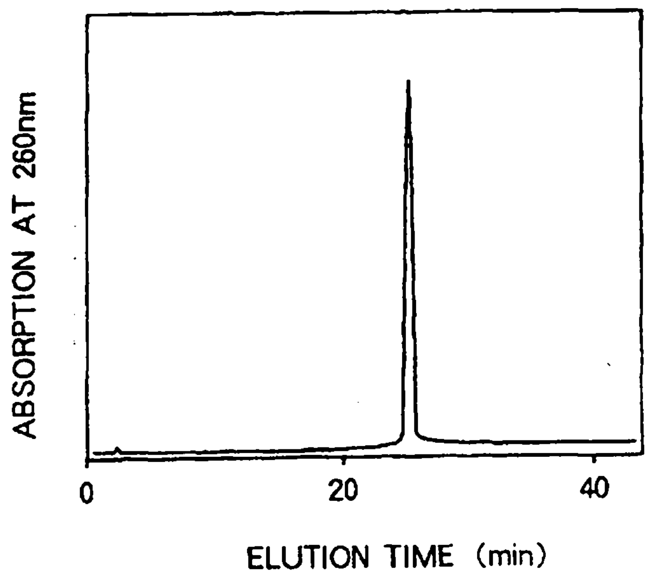
ABSORPTION AT 260nm
0
20
40
ELUTION TIME (min)

Fig. 3

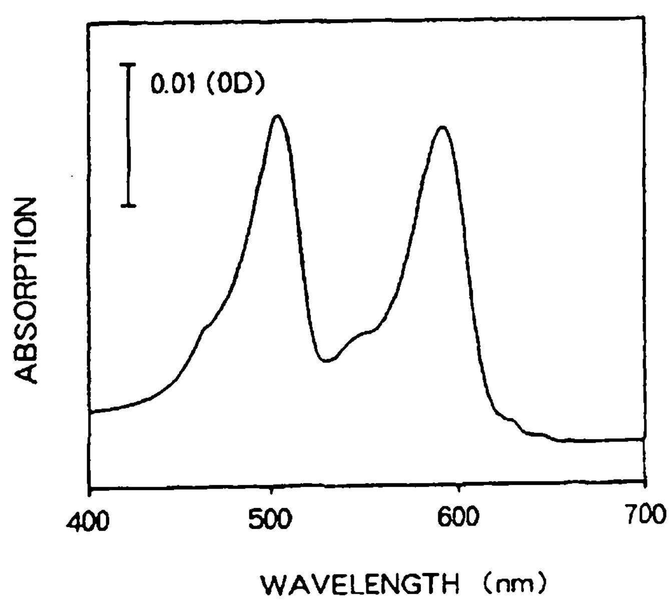
0.01 (OD)
ABSORPTION
400
500
600
700
WAVELENGTH (nm)

Fig. 4

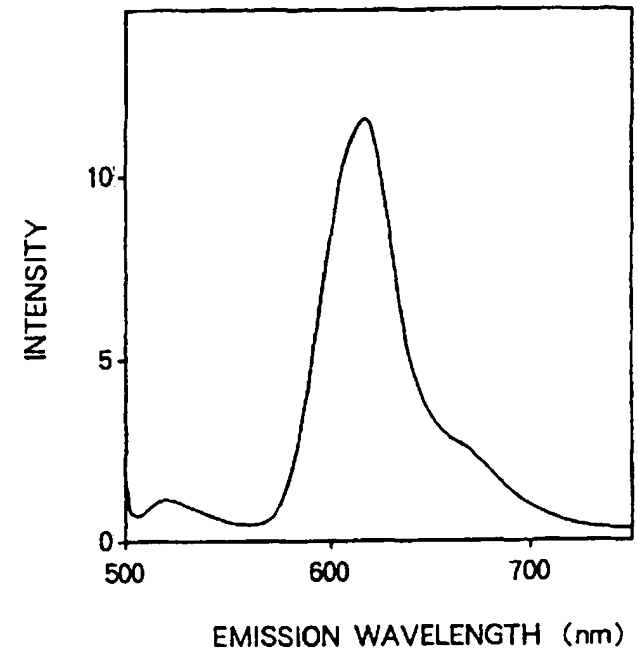
INTENSITY
10
5
0
500
600
700
EMISSION WAVELENGTH (nm)

Fig. 5

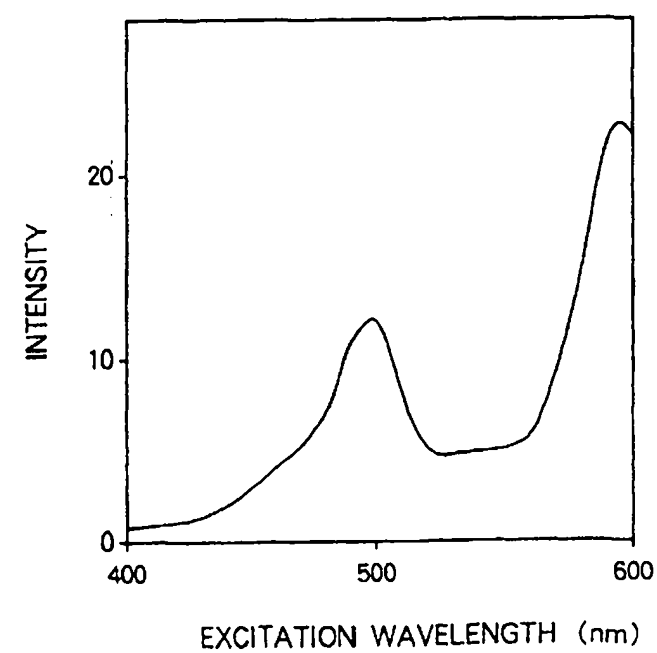
INTENSITY
20
10
0
400
500
600
EXCITATION WAVELENGTH (nm)

Fig. 6

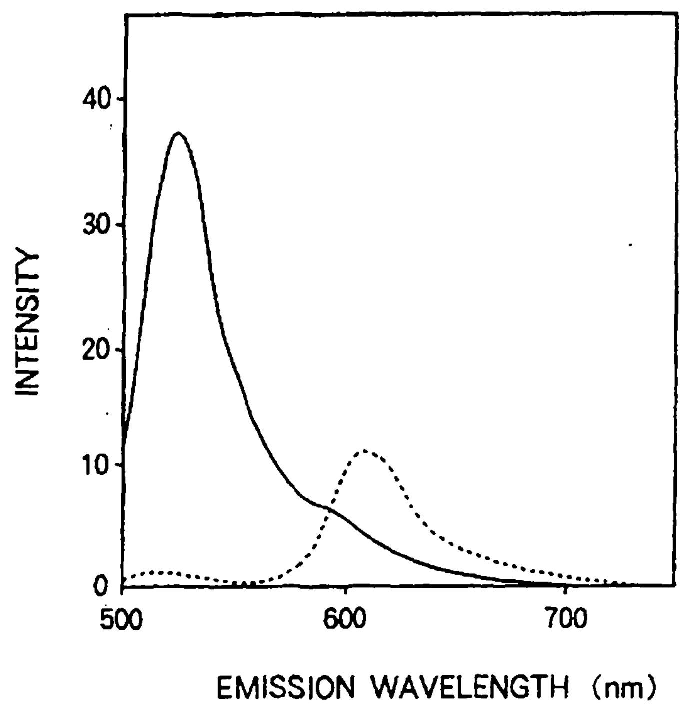

Fig. 7

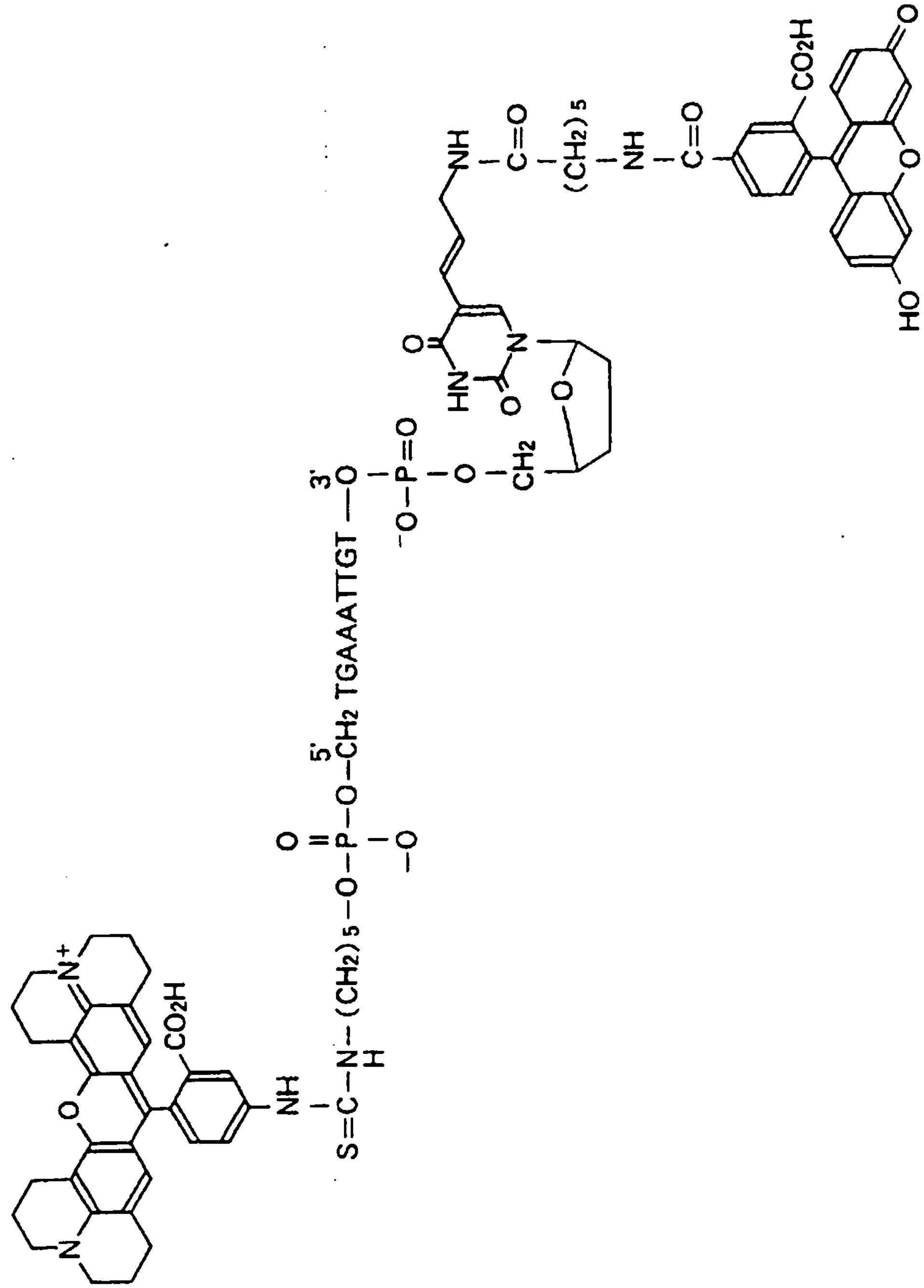

Fig. 8

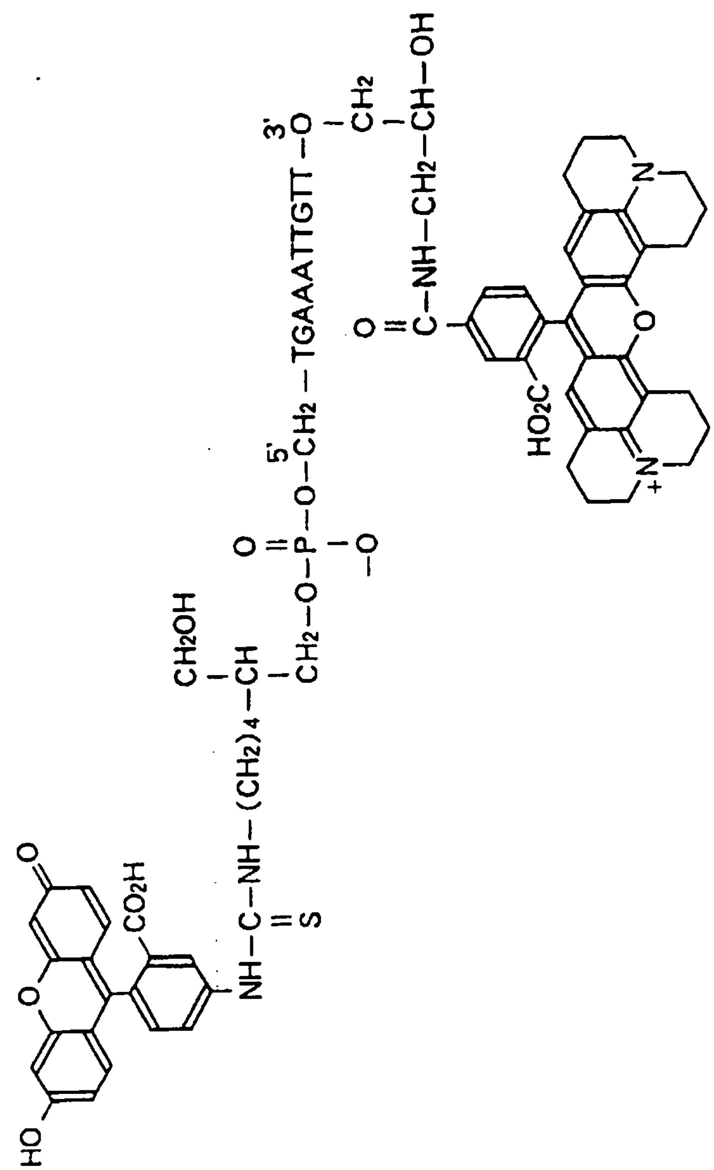

Fig. 9

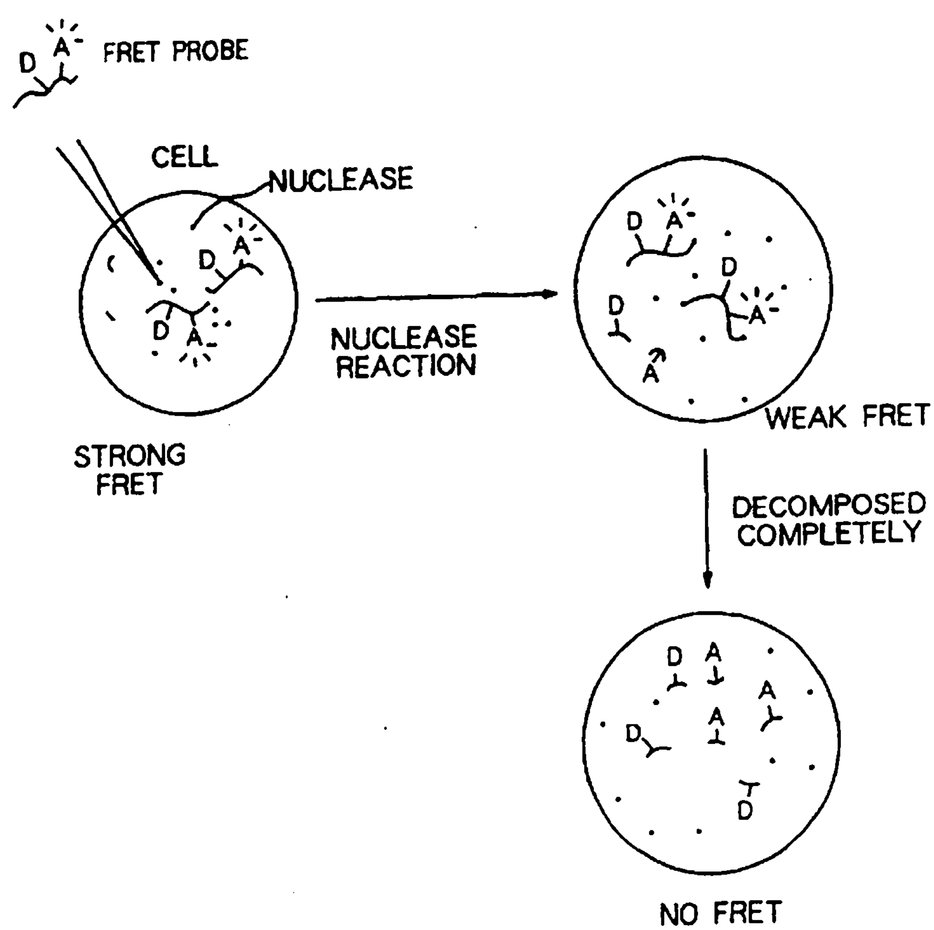
D
A
FRET PROBE
CELL
NUCLEASE
D
A
D
A
STRONG FRET
NUCLEASE REACTION
D
A
D
A
D
A
WEAK FRET
DECOMPOSED COMPLETELY
D
A
A
A
D
D
NO FRET

Fig. 10A

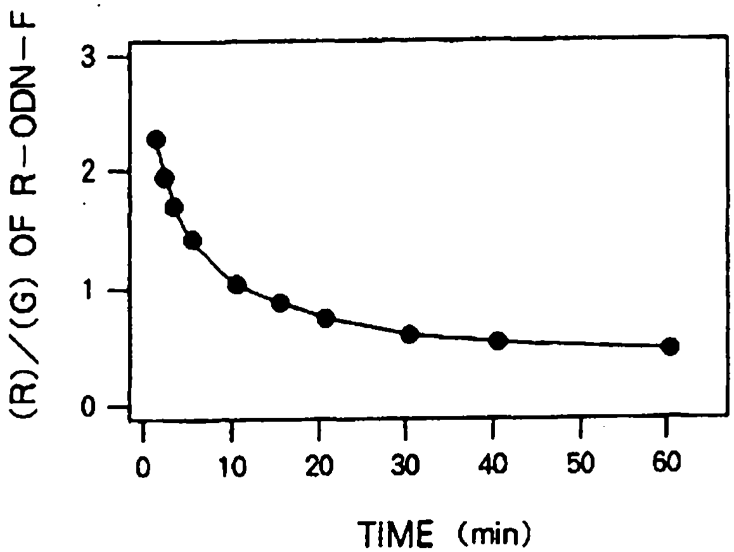

Fig. 10B